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Calpromotin, a Cytoplasmic Protein, Is Associated With the Formation of Dense Cells in Sickle Cell Anemia

R. Blaine Moore,[1]* Stephanie K. Shriver,[1] Lucy D. Jenkins,[1] Vipul N. Mankad,[2] Arvind K. Shah,[1] and Gordon A. Plishker[3]

[1]Department of Pediatrics, University of South Alabama, Mobile
[2]Department of Pediatrics, University of Kentucky, Lexington
[3]Institute for Innovative and Collaborative Projects, Sam Houston State University, Huntsville, Texas

We have tested the hypothesis that dense cell formation in sickle cell disease is associated with increased binding of calpromotin to the membrane, an event that occurs during the activation of calcium-dependent potassium transport. By SDS polyacrylamide gel electrophoresis, we found that sickle cell membranes contained more calpromotin than did normal membranes when stained with Coomassie brilliant blue or when transferred to nitrocellulose paper and immunostained with horseradish peroxidase. Also, the membranes from dense sickle cells contained significantly ($P = 0.00055$) higher levels of calpromotin, 2.62 ± 1.59 µg/mg membrane protein, compared to light sickle cells, 1.40 ± 0.70 µg/mg membrane protein, when measured by an enzyme-linked immunosorbent assay. The ratio of calpromotin associated with dense cell membranes to light cell membranes was significantly greater than 1.0 ($P < 0.00005$). Transmission electron micrographs of immunogold-labelled membranes supported the increase in calpromotin binding in dense sickle cell membranes. In addition, the immunogold probe demonstrated clustering, which was not observed in light sickle cell membranes nor in normal membranes. Finally, we incubated HbSS cells in vitro using a repetitive deoxygenation/reoxygenation procedure to produce dense cells and then measured the levels of calpromotin associated with their membranes. As expected, the levels of calpromotin bound to the membrane doubled during the procedure relative to the basal levels at the beginning of the incubation. The correlation coefficient, calculated between the increase in dense cell formation and the increase in calpromotin associated with the membrane, was statistically significant ($P = 0.038$). The results demonstrate that an increase in calpromotin binding to the membrane is associated with dense cell formation presumably through the activation of the calcium-dependent potassium channel. Am. J. Hematol. 56:100–106, 1997.

Key words: erythrocytes; sickle cell disease; calcium; potassium channels

INTRODUCTION

Persons with sickle cell disease frequently develop a population of red cells that are more dense than normal. Dense cells are important to the pathogenesis of the disease since they are directly correlated with the degree of anemia and appear to participate in vaso-occlusive events [1]. One of the mechanisms proposed for the formation of dense cells has been the loss of water and intracellular potassium and chloride due to the activation of calcium-dependent potassium transport [2–7] or potassium/chloride cotransport [8–10]. Calpromotin, a soluble cytoplasmic protein, within red cells has been associated with the activation of calcium-dependent potassium transport in red cells [11–14]. While the specific steps for activation of Ca^{2+}-dependent K^+ transport have not been elucidated, it is apparent that increased binding of calpromotin to the inner surface of the membrane is an associative event [11]. Our previous report indicated that

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*Correspondence to: R. Blaine Moore, Ph.D., FAIC, Department of Pediatrics, University of South Alabama, 2451 Fillingim Street, Mobile, AL 36617. FAX: (334) 471-7821. E-mail:rbmegm@aol.com

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this binding may result in the formation of calpromotin oligomers and that, once formed, its binding may not be easily reversed [12]. We have speculated therefore that if Ca^{2+}-dependent K^+ transport contributes to dense cell formation in sickle cell disease, then binding of calpromotin should increase in the membranes of dense cells.

In the following studies we measured the levels of calpromotin bound to erythrocyte membranes of HbSS cells and normal cells. Within the population of HbSS cells, the levels of membrane-bound calpromotin were assayed for dense cells, i.e., those cells that had undergone dehydration, and light cells of lower density. A competitive ELISA procedure and the technique of transmission electron microscopy coupled with immunogold labelling were used to assess the levels of calpromotin. In addition, we employed a repetitive deoxygenation/reoxygenation procedure to generate dense sickle cells in vitro. The levels of calpromotin bound to the membranes were measured by the ELISA procedure during the course of dense cell formation. The results confirm our hypothesis that dense cell formation in sickle cell disease is associated with increased binding of calpromotin to the erythrocyte membrane as a result of Ca^{2+}-dependent K^+ transport activation.

MATERIALS AND METHODS

Preparation of Washed Erythrocytes and Isolation of Membranes

Blood was drawn by venipuncture from normal subjects and subjects with sickle cell anemia after informed consent (written consent from sickle cell subjects) and following the approval of the institutional review board of the University of South Alabama. After centrifugation and removal of the plasma, the cells were washed four times with 0.9% NaCl solution. Membranes were prepared by lysing the cells in ice cold 5 mM sodium phosphate buffer, pH 8.0, followed by centrifugation at 15,000*g* for 10 min. The pellet was washed 3 more times with the same buffer until the membranes were white or nearly white in the case of sickle cell membranes.

Separation and Measurement of Dense Cells

Erythrocytes of normal and high density were separated on Percoll density gradients according to the procedure described by Ohnishi et al. [15]. Briefly, a 53% suspension of Percoll (Pharmacia/LKB, Piscataway, NJ) was prepared in 18% Renografin M-60 (diatrizoate meglumine, Squibb Diagnostics, New Brunswick, NJ) and 20 mM sodium HEPES buffer, pH 7.4. A continuous gradient was formed by centrifuging 10 ml of this suspension at 15,000*g* for 15 min. Either blood or a red cell suspension was layered carefully over the gradient and then centrifuged again at 2,400*g* for 45 min. Cells were removed from upper and lower layers, washed with 0.9% NaCl, and then counted in a blood cell analyzer.

SDS-Polyacrylamide Gel Electrophoresis, Western Transfer, and Immunostaining of Calpromotin

Membrane proteins were subjected to electrophoresis on 10% polyacrylamide gels containing 0.5% sodium dodecyl sulfate (SDS) according to the procedure of Laemmli [16]. Half of the gels were stained with Coomassie blue and companion gels were used for immunoblotting. Proteins from these gels were transferred to nitrocellulose paper by electroblotting. Nitrocellulose was incubated overnight in tris(hydroxymethyl) aminomethane (Tris) buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 3% bovine serum albumin (BSA). The electroblot was washed in Tris-buffered saline and incubated for 2 hr in the same buffer containing 3% BSA and a 1:10 dilution of serum from rabbits immunized against the 23,000 MW protein. The electroblot was then washed in Tris-buffered saline and incubated for 2 hr in the same buffer containing 3% BSA and a 1:500 dilution of goat anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase. Bands that had reacted with rabbit antiserum were visualized by incubating paper in a buffer containing 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.015% peroxide, and 0.5 mg/ml 4-chloro-1-naphthol.

Measurement of Calpromotin By Competitive ELISA

Calpromotin was measured by a competitive ELISA procedure as described previously [17].

Immunogold Labelling and Transmission Electron Microscopy

Membranes were incubated with rabbit antiserum to human calpromotin (1:100 dilution) for 60 min at room temperature and then washed three times with 15 volumes of 5 mM phosphate buffer pH 8 (5P8). After fixation in 5P8 buffer containing 1% glutaraldehyde, the membranes were washed twice as before and then incubated with 5P8 buffer containing 5% bovine serum albumin. A secondary biotinylated goat anti-rabbit IgG antibody was then incubated with the membranes for 30 min followed by 2 washes in 5P8 buffer and then strepavidin-gold (BRL, Gaithersburg, MD; average gold particle size = 20 nm) for 30 min at room temperature. Finally, the membranes were washed twice more, fixed with OsO_4, and then dehydrated with graded alcohol solutions. The samples were immersed in Spurr's low viscosity imbedding medium and thin sectioned on an LKB ultratome III. The sections were stained with uranyl acetate and lead citrate on copper grids and then viewed in

a Philips (Mahwah, NJ) 301 transmission electron microscope.

Formation of Dense HbSS Cells by Repetitive Deoxygenation and Reoxygenation

Washed erythrocytes from subjects with sickle cell anemia were layered over a Percoll-Renografin density gradient and then centrifuged to separate light and dense cells. The light cells were recovered and washed twice with isotonic saline and then resuspended to a 2% final volume in 20 mM HEPES buffer, pH 7.4, containing 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM NaH_2PO_4-H_2O, 7 mM glucose, 1 mM adenosine, 0.5% bovine serum albumin, and 1% v/v penicillin/streptomycin. As a control, cells were also resuspended in the same medium except that the $CaCl_2$ was substituted with 1 mM EGTA. The suspension was divided into 10-ml portions and added to 50-ml volumetric flasks. Incubation of the suspensions was performed at 37°C for varying times up to 24 hr. During this time period the suspensions were shaken in a New Brunswick (Edison, NJ) shaking water bath (model R76) and gassed with repetitive cycles of nitrogen (15 min) and air (5 min) at 100 ml/min/flask. At the end of the appropriate incubation times the cells were analyzed for the proportion of dense cells present as well as the amount of calpromotin bound to the membranes. Basal levels of calpromotin were determined from the zero time, unincubated samples, and the EGTA incubated samples.

RESULTS

Calpromotin Associated With Erythrocyte Membranes

Membrane proteins from normal subjects and subjects with sickle cell anemia were separated by SDS polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue (Fig. 1A). Because calpromotin is a minor band and to effect better resolution of this protein, the amount of total membrane protein added to the gel was increased. The calpromotin band (arrow) varies among normal subjects but appears significantly greater in all sickle cell subjects. Because more than one protein may occupy a band on one-dimensional SDS polyacrylamide gels, identical gels were run simultaneously and the proteins transferred to nitrocellulose paper. Membrane proteins were then immunostained with anti-calpromotin antibodies followed by secondary antibody conjugated with horse radish peroxidase. The results confirmed the increased calpromotin levels seen with the Coomassie staining procedure.

Calpromotin Associated With Light and Dense Sickle Cell Membranes

Since activation of the Gardös channel in normal cells has been shown to increase the association of calpromotin with the membrane, it was predicted that dense sickle cells that have undergone calcium-dependent cation depletion and water loss would be responsible for the increased calpromotin levels observed in Figure 1. To test this hypothesis, erythrocytes from sickle cell subjects were separated into light and dense cells using a Percoll-Renografin gradient procedure. Membranes were isolated from these two cell fractions and the levels of calpromotin measured by ELISA as described in Materials and Methods. Light sickle cell membranes had an average membrane level of 1.40 μg calpromotin/mg membrane protein while dense sickle cell membranes contained a significantly increased level of 2.62 μg/mg (Table I). The mean ratio of the increased calpromotin associated with dense cell membranes vs. light sickle cell membranes was 2.01. This ratio included 8 additional samples for which only the ratios could be determined. This ratio was statistically greater than 1.0. These results confirm our previous findings [11] in which the levels of calpromotin bound to normal cell membranes after

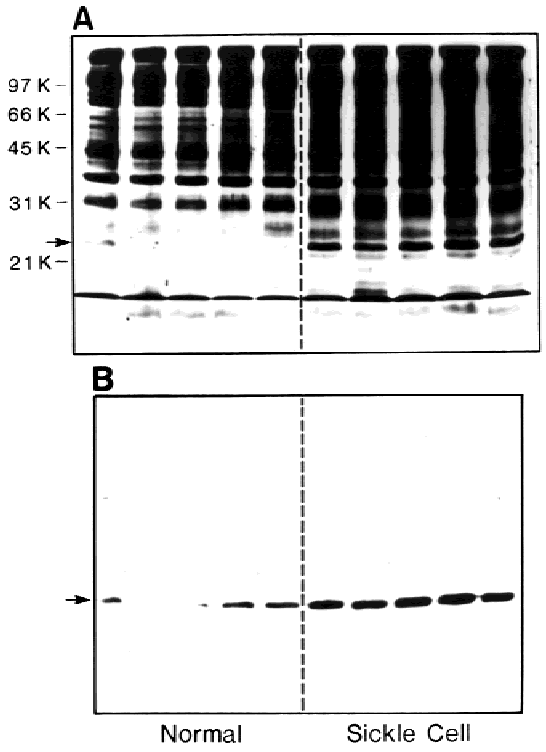

Fig. 1. Membrane-bound calpromotin in normal and sickle cell erythrocytes. Membranes were isolated from the erythrocytes of five normal subjects and five sickle cell subjects as described in Materials and Methods. The membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue (A). A duplicate gel was run and the proteins transferred to nitrocellulose paper by Western transfer. The proteins were then immunostained with anti-calpromotin antibodies followed by horseradish peroxidase conjugated secondary antibody and enzyme substrate (B). The arrow indicates the calpromotin protein band.

TABLE I. Calpromotin Bound to the Membranes of Light and Dense Sickle Cells

	Calpromotin (μg/mg)		
	Light (n = 16)	Dense (n = 16)	Ratio (n = 24)
$\bar{x}$	1.40	2.62*	2.01**
s.d.	0.70	1.59	1.04

*Calpromotin levels are significantly higher in dense cells than in light cells. $P = 0.00055$, one-sided paired *t*-test $H_o: \mu_D \leq \mu_L$.
**The mean of the ratios of dense/light cells is significantly higher than 1.0. $P = 0.000045$, one-sample *t*-test $H_o: \mu_R \leq 1$.

stimulation with calcium and its ionophore A23187 increased about 2-fold. Although the mean levels of calpromotin for light sickle cell membranes were higher than normal levels, they were not significantly different when analyzed by a Student's *t*-test for unpaired variates.

As a visual comparison of these quantitative data, we prepared transmission electron micrographs of normal membranes (EGTA or calcium), light sickle cell membranes, and dense sickle cell membranes that were stained for calpromotin using immunogold particles (Fig. 2A–D, respectively). Normal membranes prepared in the presence of EGTA showed very few gold beads, indicating low levels of calpromotin (Fig. 2A), while membranes prepared in the presence of calcium contained more beads, indicating an increased association of calpromotin with the membrane (Fig. 2B). Since calpromotin is a cytoplasmic protein, it was observed that the beads bound to only one side of a membrane, the inner surface, which appeared ''fuzzy.'' Membranes of light sickle cells (Fig. 2C) showed bead attachment that was more than normal membranes (EGTA). Finally, the membranes of dense sickle cells (Fig. 2D) demonstrated increased bead attachment over light sickle cell membranes and frequently the beads were associated in clusters of 2 or 3. It is possible that calpromotin's property of forming oligomers [18] is responsible for this clustering.

Morphometric analyses of micrographs were performed to calculate the number of immunogold particles per 100 μm of membrane for each membrane type. The results are given in Table II. Because of particle clustering, the theoretical number of calpromotin sites per 100 μ was also determined. Dense sickle cell membranes prepared in the absence of Ca^{2+} were similar to normal membranes prepared in the presence of Ca^{2+}. Also, the number of particles bound in dense sickle cell membranes was about twice the number bound to light sickle cell membranes. Even after clustering was accounted for, the number of sites on the membrane was increased in dense sickle cells over light sickle cells. These morphometric analyses confirm the observations of the micrographs and corroborate the ELISA results presented in Table I.

Calpromotin Association With Membranes During In Vitro Formation of Dense Cells

Isolated light sickle cells were transformed into dense cells by incubating them repetitively with cycles of nitrogen and air (oxygen) to induce sickling and unsickling. The association of calpromotin with the membrane was compared to dense cell formation in vitro and is shown in Figure 3. The increase in calpromotin bound to the membrane is expressed relative to the basal level, which was determined from samples not incubated or from samples incubated in the presence of EDTA. Calpromotin levels and percentage of dense cells were determined on samples in each experiment so that they could be compared directly. As seen in Figure 3, the formation of dense cells increases in a non-linear fashion and this has been verified in other experiments performed in our laboratory, which include time points between 8 and 16 hr (unpublished data). A second order polynomial regression model relating increase in dense cell formation and time produced a best significant fit ($P = 0.0041$). Note that the increase in calpromotin bound to the membrane (about 2-fold) is similar to that seen in dense sickle cells formed in vivo (Table I) and to normal cells that have been treated with calcium and the ionophore A23187 [11]. The curve for increased calpromotin binding to the membrane appeared to match closely with the formation of dense cells, and the Pearson's correlation coefficient between calpromotin bound and percent dense cells was statistically significant ($r = 0.285$, $P = 0.039$, 52df)

DISCUSSION

The results presented in this paper show that increased binding of calpromotin to the inner surface of the red cell membrane is associated with dense cell formation in sickle cell disease. This has been demonstrated by immunostaining red cell membrane proteins on nitrocellulose paper transferred from SDS-polyacrylamide gels, measuring the levels of membrane-bound calpromotin by ELISA, analyzing calpromotin bound to the membrane by immunogold labelling and transmission electron microscopy, and finally by measuring the increase in binding to the membrane with time in vitro. Our previous studies [11–14] have shown that treatment of normal cells with calcium and the ionophore A23187 results in a net loss of potassium, a decrease in mean cell volume, an increase in dense cells (unpublished data), and an increase in the levels of membrane-bound calpromotin. Furthermore, the increase in calpromotin bound to the membrane correlated significantly with the potassium that was lost from the cell as well as the cellular dehydration over the external calcium concentration that was used. The internal calcium concentrations in these ex-

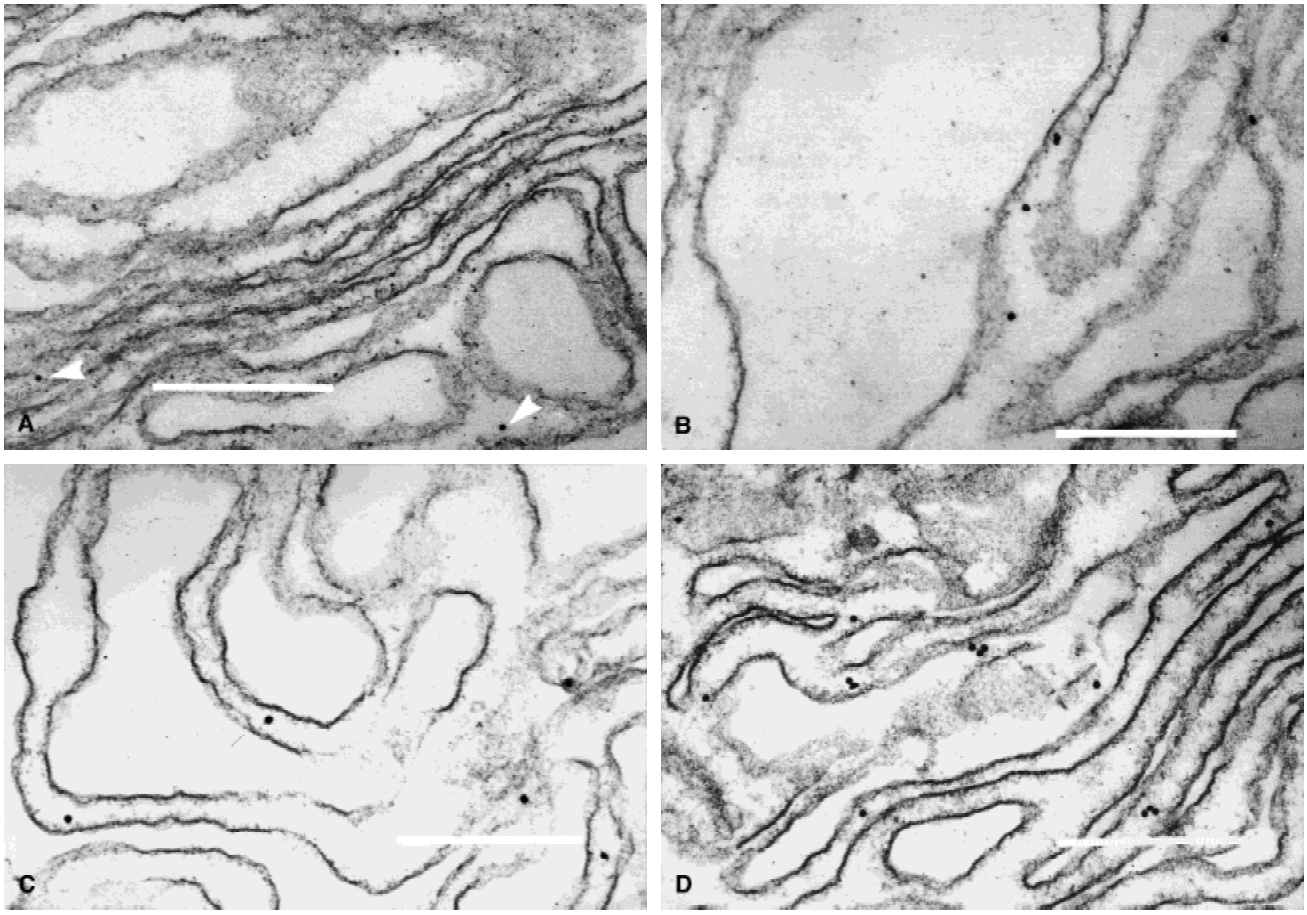

Fig. 2. Transmission electron micrographs of immunogold-labelled calpromotin bound to membranes of light and dense cells from a subject with sickle cell anemia. Arrowheads indicate the location of immunogold particles. A: Membranes derived from normal erythrocytes prepared in EDTA. B: Membranes derived from normal erythrocytes in the presence of 0.1 mM $CaCl_2$. C: Membranes derived from light sickle cells. D: Membranes derived from dense sickle cells. Bars-500 nm.

TABLE II. Calpromotin Bound to Normal and Sickle Cell Membranes: Morphometry of Electron Micrographs

Membrane source	Gold particles per 100-μm membrane	Sites per 100-μm membrane
Normal cells (EGTA)	11	11
Normal cells (Ca^{2+})	76	55
Light sickle cells	35	35
Dense sickle cells	78	50

periments were not measured but it is expected that they were considerably lower, i.e., in the nanomolar range, as reported previously [19–21]. There is now a strong body of evidence demonstrating that calpromotin plays a role in the activation of calcium-dependent potassium transport in human red cells [11,13,14]. As a consequence of these results, we propose that the formation of dense cells in sickle cell disease involves, at least in part, an activation of calcium-dependent potassium transport and that this process occurs in concert with increased association of calpromotin with the membrane.

Over the years there has been considerable evidence to support a role for intracellular (cytoplasmic) calcium in sickle cell dehydration. Throughout the 1970s there were several reports of a calcium abnormality in HbSS cells. Dense cells formed in vivo or in vitro had higher levels of calcium than normal [22–24]. Deoxygenated sickle cells took up 2.6 times more calcium than normal cells or control cells during a 2-hr incubation [25,26]. The ability of HbSS cells to extrude preloaded calcium was abnormal [25,27], leaving a residual level of calcium within the cell, which could only be removed by the ionophore A23187. Controversy arose, however, when it was shown [28,29] that most, if not all, of the increased intracellular calcium was sequestered within endocytic vesicles and was not cytoplasmic. Indeed, when the cytoplasmic ionic calcium concentration in sickle erythrocytes was measured using an NMR probe [30], it was found to be within the normal range (10–30 nM). These measurements were made under static conditions and the possibility remained that the ionic calcium concentrations rose transiently during sickling. Using the calcium-dependent fluorescent probe, *benz-2,* Etzion et al. [19]

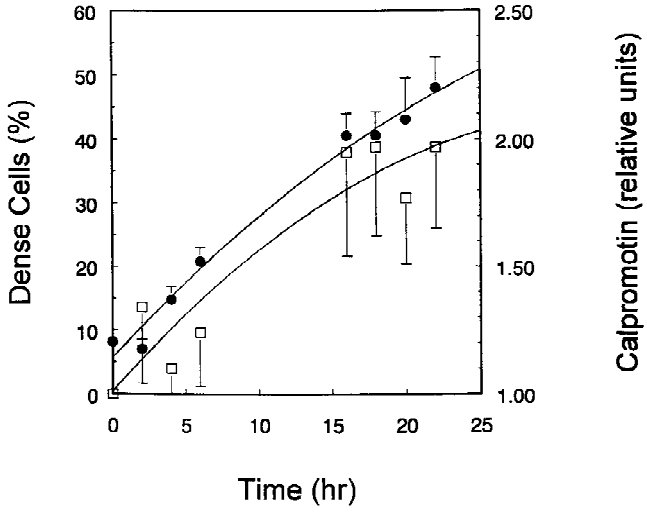

Fig. 3. Dense cell formation and calpromotin binding to the membrane during in vitro repetitive deoxygenation/reoxygenation cycles. Isolated low density HbSS erythrocytes were incubated as described in Materials and Methods and then evaluated for percentage of dense cells formed (●) and levels of calpromotin bound to the membrane (□). The same cell suspensions within each experiment were used to measure both parameters to allow correlation analyses to be performed. The values show the means ± SE from 6 separate experiments.

demonstrated that during deoxygenation HbSS erythrocytes increase their intracellular ionic calcium concentrations some 2- to 4-fold. Coupled with a decreased calcium pump activity, the authors concluded that these events might be sufficient to raise the intracellular calcium concentrations during sickling to activate the calcium-dependent potassium channel. As final evidence, inhibitors of Ca^{2+}-dependent K^+ transport such as clotrimazole [6,31], charybdotoxin [15], and quinine [32,33] all inhibit dense cell formation in vitro, and for clotrimazole in vivo [34], establishing a role for this transport system in sickle cell dehydration.

It is possible that not all HbSS cells undergo dehydration by this mechanism since the red cell population in sickle cell disease is very heterogeneous with a short life span and with an elevated reticulocyte pool. Reticulocytes possess an active potassium/chloride cotransport [35–37] (preferentially to mature cells) and it has been proposed that some of these cells dehydrate by this alternate mechanism. In a recent report, Franco et al. [38] investigated both potassium efflux pathways in transferrin receptor positive cells (a fraction of reticulocytes) and found that both calcium-dependent potassium efflux and potassium/chloride cotransport contributed to the dehydration of these cells. Studies in our own lab (unpublished data) showed that a portion of light cells (20–30%) will dehydrate during the in vitro repetitive sickling procedure in the absence of calcium (1 mM EDTA), suggesting a mechanism other than Ca^{2+}-dependent K^+ transport. However, the membrane-bound calpromotin levels of these cells were equivalent to calpromotin levels in control cells that were not incubated, indicating specificity of calpromotin for calcium-dependent dehydration.

In conclusion, the results of this study support a role for calpromotin binding to the membrane during dense cell formation in sickle cell disease through the activation of the Ca^{2+}-dependent K^+ channel.

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